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13. ABSTRACT (Maximum 200 words)

The Air Force employs or generates a variety of materials, such as fuels, some of which may be potentially hazardous and could be released into the environment. Effective tools are required to predict and assess the fate of these agents in the environment. It is equally important, to understand the basic biological mechanisms of these agents. We have thus begun to assess whether these fuels kill cells via scheduled cell death. During the past granting period we have developed a relatively simple, inexpensive, and easy-to-use diagnostic tool for detecting whether a cell's DNA has been damaged by fuels. We have also performed the initial basis for an understanding of a key enzyme (apopain) which is involved in the cleavage of poly(ADP) ribose polymerase (PARP) during apoptosis. We feel that both contributions have potential applications for the screening of the toxicity of newly developed fuels intended for U.S. military use.

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FINAL REPORT

THE KEY INVOLVEMENT OF POLY(ADP-RIBOSYL)ATION IN DEFENSE AGAINST TOXIC AGENTS: MOLECULAR BIOLOGY STUDIES

(AFOSR – 89 – 0053)

June 1, 1998

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FINAL REPORT
(June 1, 1998)

1.Initial Studies On Parp Cleavage During Apoptosis By "Apopain" And Its Inhibition

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., Munday, N.A., Raju, S.M., **Smulson, M.E.**, Yamin, T.T., Yu, Y.L., and Miller, D.K. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. (Nature. **376**: 37-43. 1995).

My involvement in this new aspect of PARP cell biology began as a collaboration between my laboratory and the Department of Medicinal Chemistry, Merck Research Laboratories of which Dr. Donald Nicholson is the head. The resulting data indicated a potentially pivotal role for PARP destruction in apoptosis, and forms the basis for our renewed proposed studies utilizing this function of PARP to determine the mechanism of genotoxicity of fuels such ADN, and JP-8. As noted earlier, genetic evidence in the nematode *C. elegans* implicates the product of the CED-3 gene, which encodes a cysteine protease related to mammalian interleukin-1b converting enzyme (ICE), in the control of apoptotic cell death. One of the potential targets for the human equivalent of CED-3 has been identified as PARP which is proteolytically inactivated at the onset of apoptosis by a protease related, but not identical to, ICE.

The resulting cleavage between Asp 216 and Gly 217 separates the N-terminal DNA-nick sensor of PARP from its C-terminal catalytic domain (Fig. 2). Using the full-length human cDNA clone, pcD-12, isolated in my laboratory, [^{35}S] PARP was generated as a substrate by in vitro transcription/translation and was then combined with various cell extracts (Fig. 1). We utilized a human osteosarcoma cell line with the property of spontaneous apoptotic death. This contains substantial PARP cleavage activity that is markedly higher in extracts from apoptotic cells versus non-apoptotic cells (lanes 2 and 3, respectively). The cleavage site (Fig. 2) within PARP (DEVD216--G217) was identified and represents a separation of the two zinc-finger DNA-binding motifs in the amino terminus of PARP, from the automodification and catalytic domain of the enzyme rendering this protein totally inactive in the presence of the massive DNA strand breaks (Fig. 3) which occur during apoptosis. Upon reaching confluency in culture, osteosarcoma cells undergo morphological and biochemical changes characteristic of programmed cell death, including internucleosomal DNA cleavage (Fig. 3). They can also be induced to apoptosis by both chemicals and ionizing radiation. The PARP cleavage activity, measured in cell extracts, was elevated more than ten-fold (Fig. 4). (*an assay that will be used Aim I).* Inspection of Fig. 4 shows the key observation that PARP cleavage activity maximizes on day five, just prior to the extensive internucleosomal DNA cleavage (Fig. 3) which occurs after this time in this system. Normally, such extensive DNA strand breaks would highly stimulate PARP activity in cells and cause total depletion from cells, almost instantaneously, of its NAD and perhaps its ATP pools.

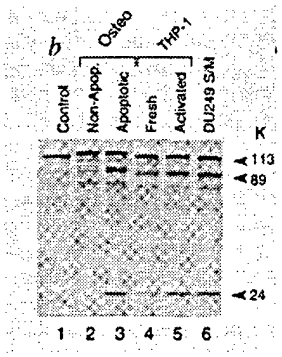


Figure 1

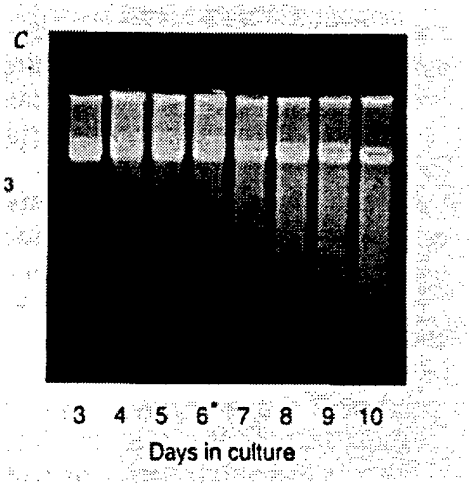


Figure 3

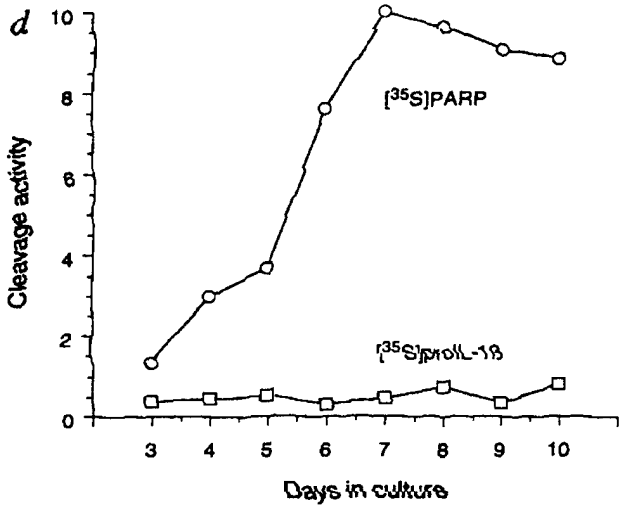


Figure 4

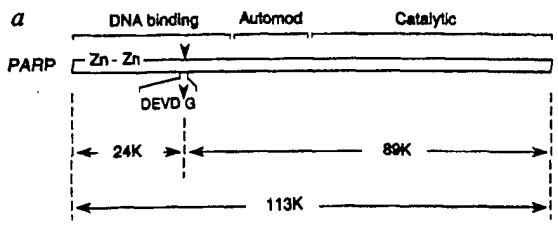


Figure 2

We next explored the sequences proximal to the Asp 216, Gly 217 bond as a template for inhibitor drug design. A tetrapeptide aldehyde containing the P_1-P_4 amino-acid sequence of the PARP cleavage site (DEVD 216-G 217) was synthesized (Ac-YVAD-CHO). It was found that Ac-YVAD-CHO inhibits the PARP cleavage activity in cell extracts with a 50% inhibitory concentration (IC_{50}) of 0.2 nM. The tetrapeptide aldehyde containing the proIL-1b recognition sequence for ICE (Ac-DEVD-CHO) was not inhibitory (Fig. 5). Accordingly, this very highly specific inhibitor will be available for a number of the studies outlined in the proposal below. We also showed that the cowpox-virus serpin CrmA, which is a very potent inhibitor of ICE, was a very **poor** inhibitor of PARP cleavage activity.

There are at least five, if not more, known human enzymes in the ICE/CED-3 family of cysteine proteases. CPP-32 is the most closely related of the animal-human enzymes. We chose the human monocytic leukemia cell line, THP-1 cells, from which ICE was originally purified, in order to identify the specific enzyme responsible for PARP inactivation. To **selectively** purify PARP cleavage activity two biotinylated derivatives of the Ac-DEVD-CHO tetrapeptide aldehyde inhibitor were synthesized and used as affinity ligands for the PARP cleavage enzyme. SDS-PAGE showed that the purified PARP cleavage enzyme was composed of two major polypeptides of approximate M_r 17K and 12K (see lane 5, 6, Fig. 6).

The sequence alignments of purified apopain subunits K 17 and P 12, as determined by electrospray mass spectroscopy at the Merck Laboratories, in comparison with the five members of the ICE-like family is shown in Fig. 7. The N-terminal sequence of the 12K subunit of the purified PARP cleavage enzyme corresponds with that of CPP-32b.

In addition, a continuous fluorometric assay for apopain using the substrate Ac-DEVD-AMD (amino-4-methylcoumarin) was developed. **This assay will also be utilized in Aim I to assess the effects of fuels such as ADN and JP-8 on cellular toxicity, via an apoptotic type of mechanism.** Cleavage of this substrate by apopain showed Michaelis-Menten kinetics with a K_m value of 9.7 ± 1.0 μM.

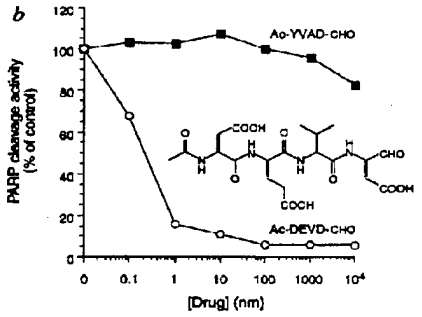
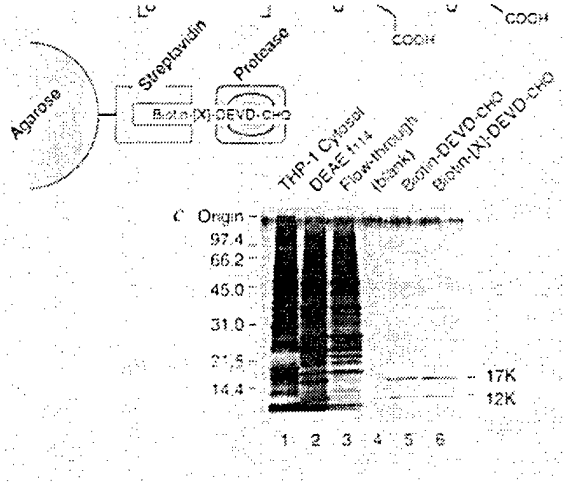


Figure 5



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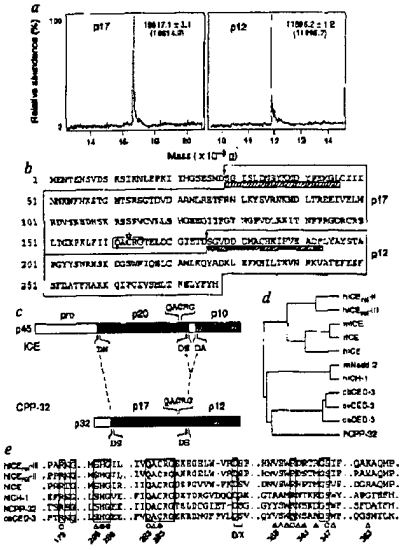


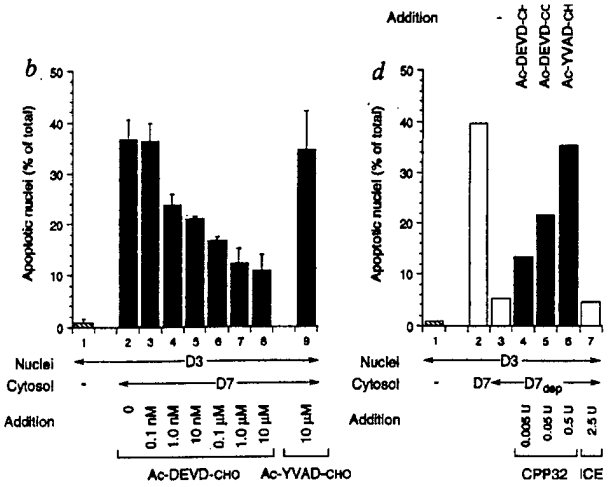
Figure 7

In this study, a very convenient second *in vitro* assay (Fig. 8) for apoptosis was established. We utilized osteosarcoma nuclei from non-apoptotic (day 3) cells. Apoptotic-typical chromatin changes in the nuclei were measured using Hoechst 33342. The data shown in Fig. 8B indicate that the cytosols from non-apoptotic osteosarcoma cells had little effect on nuclear morphology, whereas those from progressively apoptotic

cells were capable of inducing apoptosis-like changes in the nuclei. The degree of apoptotic morphology was consistent with the contents of apopain, as shown earlier in Fig. 4. In Fig. 8A, we utilize naive apoptotic nuclei from day 3 and as a source of apopain and other factors cytosol from day 7, which showed the highest apoptotic activity in Fig. 8. We observed that the peptide inhibitor, Ac-DEVD-CHO, which prevents PARP cleavage, significantly reduces the ability of nuclei to undergo the morphological changes associated with apoptosis. The use of this inhibitor would be useful in conjunction with exposure of cells or tissues either ADN or JP-8 vapor fumes or other agents of interest to AFOSR to clarify whether these agents, in part, initiate apoptotic events in various types of tissues.

It is important to note that the peptide Ac-DEVD-CHO would be expected to also inhibit the cleavage by apopain of other substrates for apopain/Yama such as the catalytic subunit of DNA-PK and U1-70 kD, etc., and thus lack of cleavage of these equally significant substrates may, in fact, represent the major reason for inhibition of apoptosis in this assay.

Figure 8a



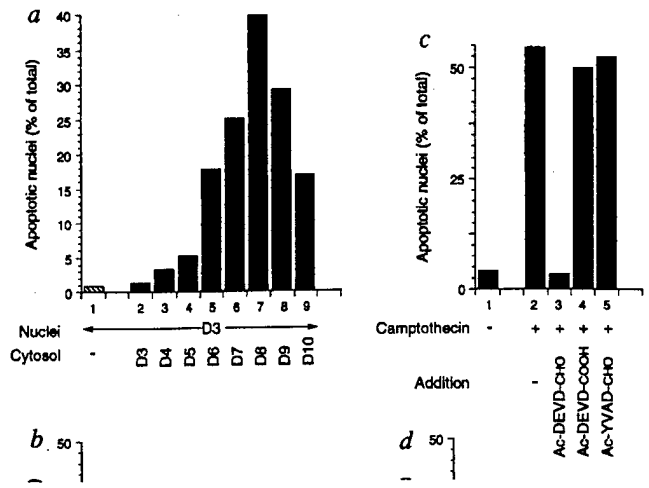


Figure 8b

Rosenthal, D., Ding, R., Simbulan-Rosenthal, C.M., Vaillancourt, J.P., Nicholson, D.W., and **Smulson, M.**: Intact Cell Evidence for the Early Synthesis and Subsequent Late Apopain-Mediated Suppression of poly(ADP-ribose) During Apoptosis. Experimental Cell Research. **232**: 313-321 (1997).

Most of the early research on PARP-cleavage and apoptosis, including our own studies, have relied on *in vitro* assays or immunoblot analysis of PARP cleavage or apopain activity. It, therefore, seemed expedient at this stage to explore PARP activity and cleavage in the osteosarcoma cell model of apoptosis by examining the various participants in this aspect of programmed cell death by immunofluorescence in the **context** of the **whole cell**.

In this recent progress cytosolic fractions of cells incubated for various times in culture were assayed for PARP-cleavage activity by the assays developed above (Fig. 3) to confirm that the proenzyme CPP32b was activated. Subsequently, human cells grown on coverslips were incubated for up to 10 days and fixed at daily intervals for examination of nuclear poly (ADP-ribosyl)ation with a guinea pig antiserum to PAR **polymer**. We did not detect the presence of polymer after 24 h of culture, indicating the absence of DNA strand breaks, PARP, or both (Fig. 9). After 2 days of culture, substantial amounts of poly(ADP-ribose), not observed earlier, were detected within the nuclei of all attached cells. After 3 days, the nuclei of all attached cells stained intensely for the polymer (Fig. 9). DNA analysis did not indicate any internucleosomal cleavage at this time point (Fig. 3). However, recently, Neamati et al. demonstrated large DNA fragments (1 Mb) and lamin B1 degradation early in apoptosis, prior to any evidence of internucleosomal cleavage. In these results, the amount of polymer was observed to be

markedly reduced after four days. Subsequently, only a faint diffuse staining pattern was detected, even though substantial DNA fragmentation had been shown during this period (Fig. 9). If intact PARP **were** present in cells during these latter time points, polymer would be expected to be very elevated and NAD and ATP levels would also be expected to be almost totally depleted.

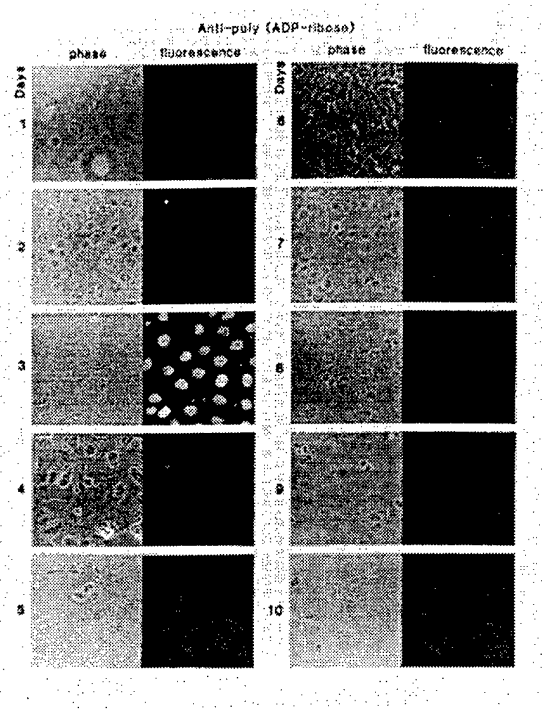


Figure 9

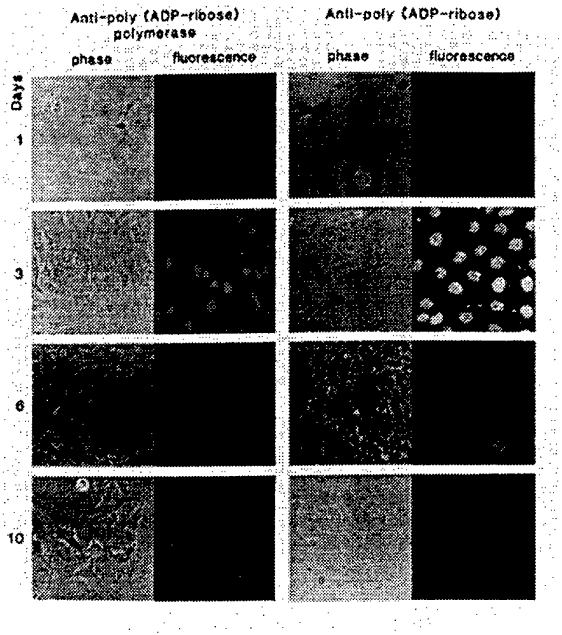


Figure 10

To determine whether the time course of expression of polymer reflected that of the PARP protein, we subjected the cells to immunofluorescence analysis with antibodies specific for full length PARP. While PARP concentrations have been observed to be high minimally in a number of cell types, the time course of PARP expression (Fig. 10, left) was similar to that of the polymer. Surprisingly, only small amounts of PARP were detected after 1 day in culture, perhaps merely reflecting a time lag after plating of the cells. However, a substantial amount of PARP was detected in the nuclei of all cells after 2 days. An unanswered question to be addressed in other PARP projects in my laboratory is whether there is an authentic biological function(s) involving apoptosis for PARP expression at this specific time.

PARP staining showed a punctate pattern throughout the nucleus. Staining is more intense in perinucleolar regions, but is excluded from the nucleoli. The intensity of PARP staining gradually decreases between days 3 and 10, although weak staining is still apparent after 10 days. This progress during the last period support the concept that nuclear disruption involving strand breaks is present early in the time course of slow apoptosis, before morphological changes and before the appearance of the characteristic nucleosome ladder and that PARP is essentially totally cleaved. During the last year, there have been several other observations by colleagues (as yet unpublished) very similar to ours showing an activation of PARP activity early in apoptosis.

In Figs. 2 and 3 above, we showed by *in vitro* assays, that apopain catalyzed PARP cleavage occurs. To confirm now that this cleavage occurs in intact cells, the cells were treated with antibodies generated against the peptide corresponding to the DNA binding domain (DBD) of PARP. Our preliminary in the context of the whole cell, (Fig. 11, left panel) shows by immunofluorescence analysis, that the PARP DBD (24 k Da) human osteosarcoma cells, appears only after 6 to 7 days, at the time that the abundance of PARP and poly(ADP)R polymer is decreasing.

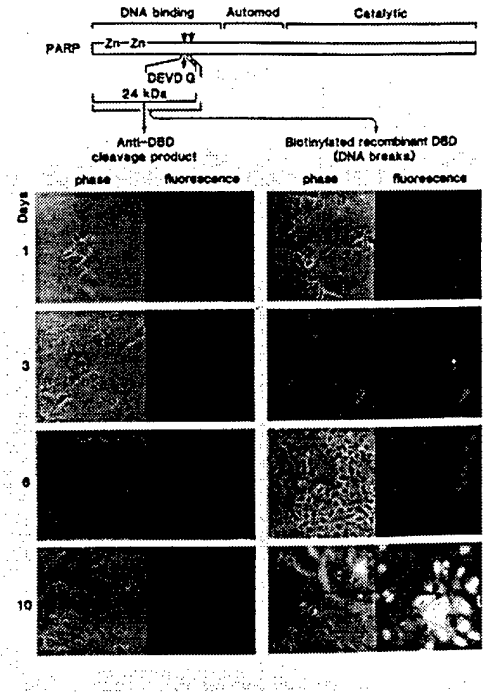


Figure 11

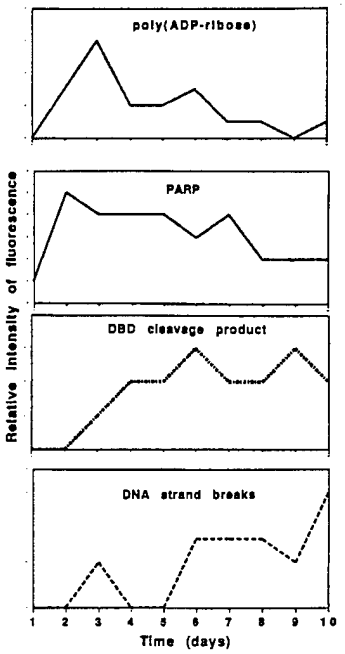


Figure 12

To avoid the use of antibodies to detect the PARP DBD bound to the DNA stand breaks in osteosarcoma cells, we conjugated the bacterially-expressed DBD to biotin to allow detection via reaction with streptavidin and ECL. These preliminary experiments assessed the levels of DNA strand breaks in the fixed intact cells by staining with this reagent (Fig. 1, right hand panel). By day 10, the nuclei of many cells stained intensely (consistent with the data of Fig. 3). Significant stand breaks were also detected from days 6-9.

The normalized results from several preliminary experiments of the four essential PARP-related biological participants during the entire time period of apoptosis, obtained from the relative fluorescence intensities of Figs. 9-11, are displayed graphically in Fig. 12. Taken together, the data emphasizes, in the **context** of the **intact cell**, that poly(ADP-ribosylation) is **inconsistent** with the late stages of apoptosis.

Rosenthal, D.S., Ruchuang, D., Simbulan-Rosenthal, C.M.G., Cherney, B., Vanek, P., and Smulson, M.; "Detection of DNA breaks in apoptotic cells utilizing the DNA-binding domain of poly(ADP-ribose) polymerase with fluorescence microscopy," Nucleic Acid Research **25**: 1437-1441 (1997).

The new **biomarker** utilizing straining on fixed cells with the biotinylated PARP DNA binding domains was developed during the last funding period. Many of the currently available methods for examining DNA strand breaks *in situ* rely on the ability of exogenous enzymes such as DNA polymerase or terminal transferase to add labeled

dNTPs to the 3'-OH ends of the strand breaks and subsequent detection of the incorporated nucleotides by immunofluorescence microscopy. We reasoned that the DBD of PARP might provide a more sensitive probe for DNA strand breaks that would eliminate the requirement for the often-labile enzymes and nucleotide substrates.

Clone pCD12, containing the full-length cDNA encoding human PARP in an Okayama-Berg vector, was used as a polymerase chain reaction (PCR) template for construction of a PARP DBD expression vector. PCR was performed with (i) a 28-bp primer that contained a Bam HI restriction site upstream (nucleotides 164 to 180) and (ii) a 22-bp primer that contained a Hind III restriction site downstream (nucleotides 837 to 854) of PARP cDNA. The PARP cDNA fragment thus amplified encompassed the region that encodes the two zinc fingers of the enzyme as well as the KKKSKK nuclear localization signal. Amplification was performed for 21 cycles, and the product was then ligated into the bacterial protein expression vector pQE30 (Qiagen). The DBD of PARP was subsequently expressed in *E coli*, and purified to more than 95% homogeneity by affinity chromatography using a Ni-NTA column (Fig. 13). The PARP DBD fusion protein was recognized on immunoblot analysis by polyclonal antibodies, obtained subsequently, to this region of PARP

To avoid the use of antibodies to detect the PARP DBD bound to the DNA strand breaks, we conjugated the bacterially expressed DBD to biotin so as to allow detection by reaction with horseradish peroxidase-conjugated streptavidin and enhanced chemiluminescence (ECL). We first tested the modified DBD detection system in two human B cell lines that are known to undergo apoptosis via endonuclease cleavage of DNA following serum depletion, unlike normal B cells which become quiescent upon serum withdrawal.. Apoptosis was induced in either a B cell line immortalized with EBV *in vitro* (Fig. 14), or in Burkitt Lymphoma-derived B cells (Fig. 15) by withdrawal of autocrine growth factors.

In virtually all instances, only those cells showing the morphological characteristics (cell shrinkage and nuclear condensation) of apoptosis were stained by the biotinylated PARP DBD. The number of stained cells increased with time after autocrine factor withdrawal.

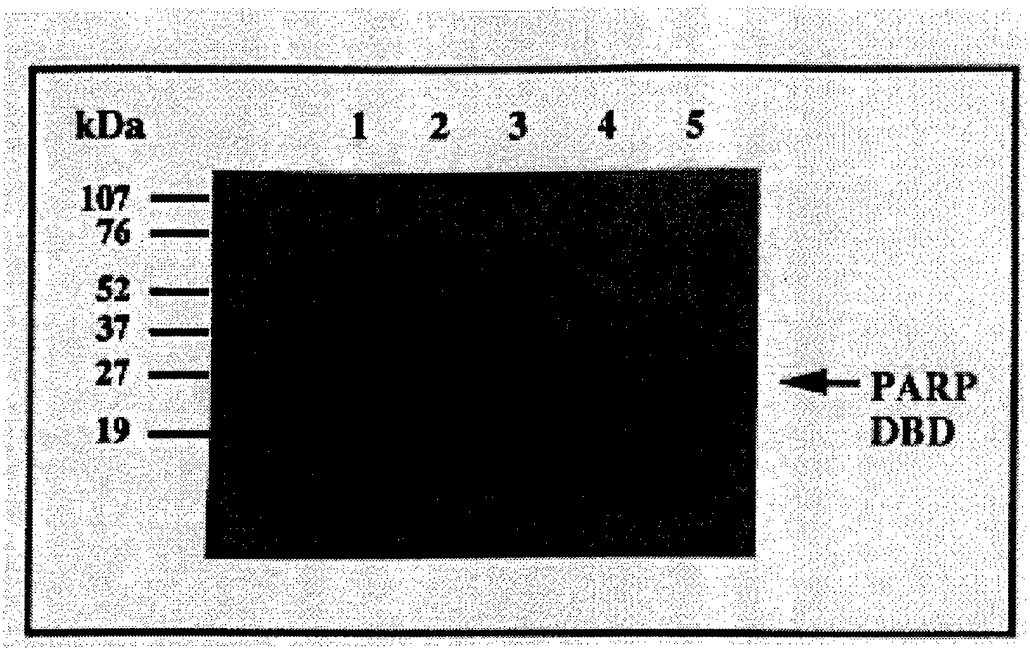


Figure 13

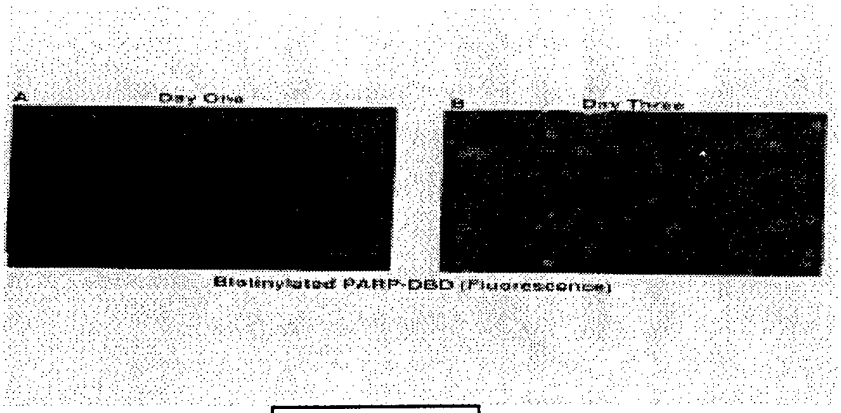


Figure 14

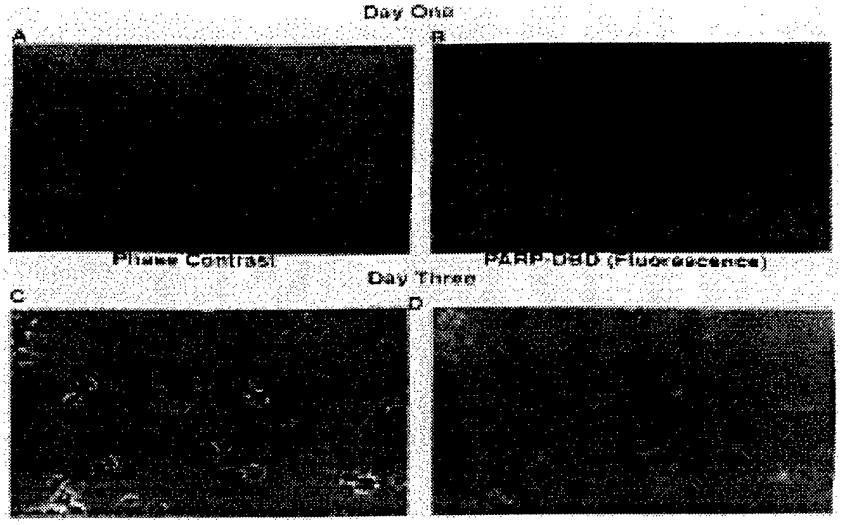


Figure 15

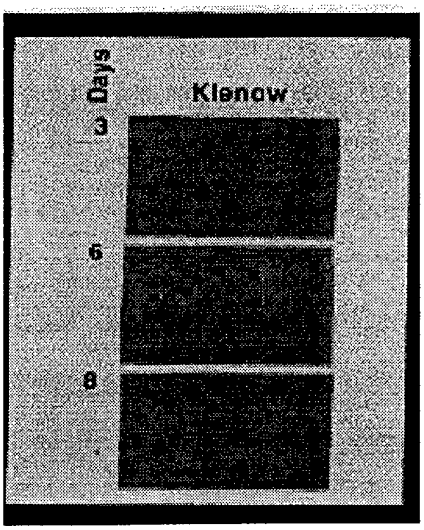


Figure 16

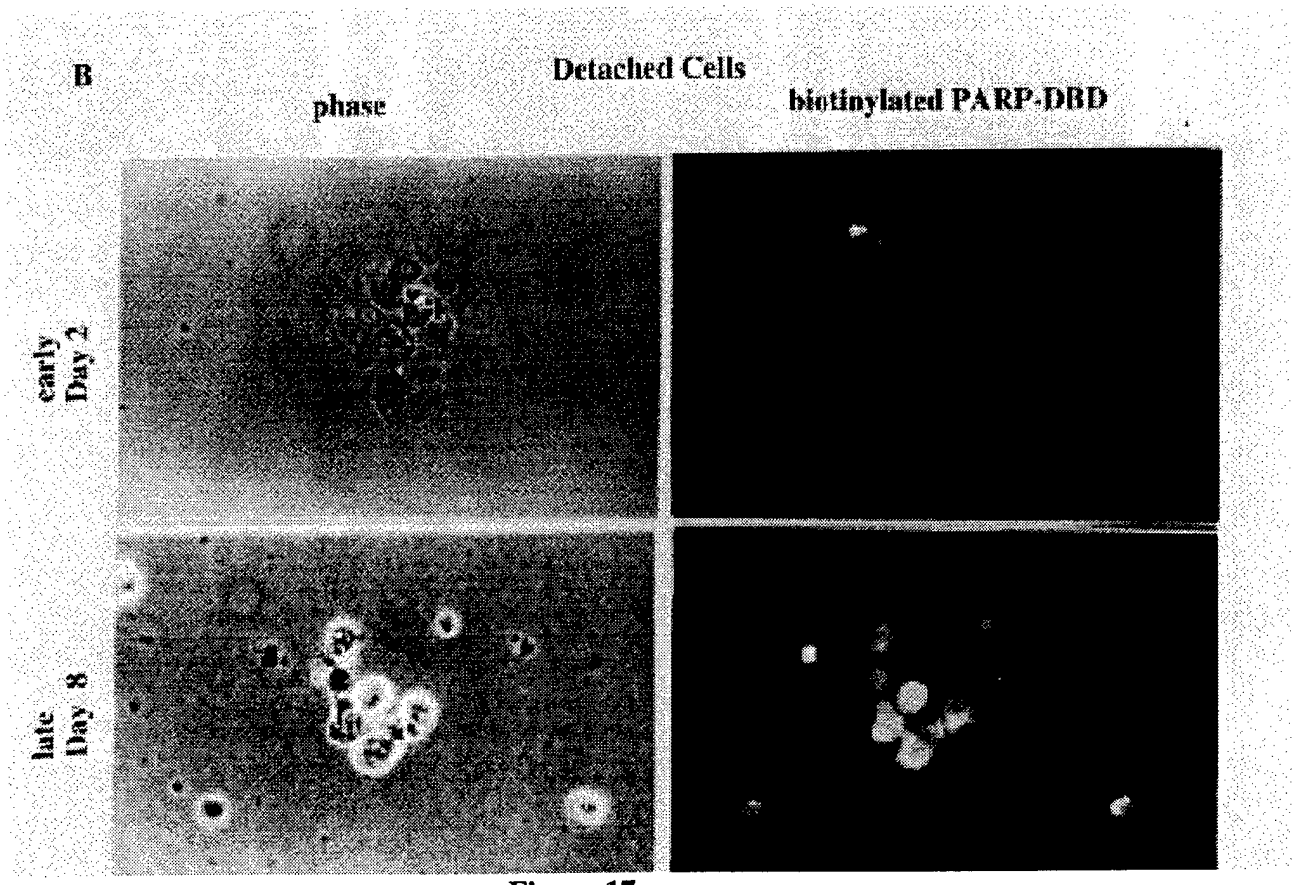


Figure 17

In this new work summarized above, we characterized the stages of apoptosis with respect to PARP activation, PARP proteolysis, and DNA fragmentation, in the same human osteosarcoma cell line that undergoes a slow (8 to 10 days), spontaneous, and reproducible death program in culture used in the work published in *Nature*. The activation of PARP (shown above in Fig. 9,10) occurred early, within 2 days of cell plating for apoptosis, while PARP proteolysis was detected immunocytochemically between 4 and 6 days. DNA fragmentation was not evident until 6 to 10 days in culture, as determined by nucleosome ladder formation (Fig. 3). In the current study, we wished to compare the sensitivity of this new methodology for detecting strand breaks, using the osteosarcoma system, to a widely used technique. We first confirmed that the osteosarcoma cells were undergoing apoptosis, as measured by apopain activity. We then tested a well-established assay for DNA strand breaks in apoptotic cells which relies upon the ability of the Klenow fragment of DNA polymerase I to incorporate nucleotides *in situ* . We therefore measured the levels of DNA strand breaks in the fixed cells by incubating the human osteosarcoma cells with Klenow enzyme in the presence of biotinylated nucleotides. Samples were analyzed from early (day 3), middle (day 6), and late (day 8) stages of apoptosis; the result of a typical experiment is shown in Fig. 16. In general, the number of osteosarcoma cell nuclei positive for *in situ* nucleotide incorporation also increased with time, consistent with our other assays for apopain

activity and DNA strand breaks. However, fewer apoptotic nuclei were detected by the Klenow assay than by the PARP-DBD assay (Fig. 17). At day 2, none of the nuclei were stained. By day 6, several nuclei that appeared morphologically apoptotic stained positively for nucleotide incorporation. More nuclei stained positively for strand breaks by day 8, although the proportion of positive cells remained in the minority. At this time point, <30% of attached cells were Klenow-positive compared to >80% that were DBD-positive (Fig. 17).

Bowen C., Voeller, H., Rosenthal, D., Bershem, G., Manon, B., **Smulson, M.E.**, and Gelmann, E.P. Antisense Oligonucleotides for Cysteine Proteases CPP-32 and ICE-LAP3/Mch3 Inhibit Apoptosis in Prostate Cancer Cells. Submitted to Cell Growth and Differentiation. 1996.

Apoptosis in PC-3 prostate cancer cells can be induced by 50 μM etoposide, and inhibitor of topoisomerase II. Within twelve hours of etoposide treatment, levels of mRNA for two closely related ICE-like proteases, CPP-32 and ICE-LAP3, are elevated approximately ten fold. Within 24 hours cysteine protease activity is induced as **demonstrated by cleavage of polyADP-ribose polymerase**. By 72 hours the majority of cells demonstrate *in situ* end labeling for DNA fragmentation and by 120 hours 25% of the cells bind annexin V to phophatidylserine molecules that have appeared on the exterior of the plasma membrane. To assess the importance of elevated cysteine protease mRNA levels seen at 12 hours, we treated cells with antisense oligonucleotides to one, another, or both cysteine proteases. Antisense oligos to CPP-32 inhibited DNA end-labeling by 50%. Although antisense to ICE-LAP3 alone had no effect on apoptosis, in combination with antisense to CPP-32, there was 70% inhibition of DNA end-labeling. Antisense oligonucleotides to ICE-LAP3 and CPP32 had no effect. Our results show that in PC-3 cells synthesis of CPP32 and ICE-LAP3 is important for subsequent DNA fragmentation during apoptosis.

2. Elucidation of the biological function(s) of PARP by the expression of antisense PARP transcripts in HeLa and 3T3-L1 cells

One major past aim of this AFOSR project was to establish and characterize stably transfected cells with antisense cDNA to PARP driven by an inducible promoter and to establish conditions where depletion of PARP could be achieved, only upon induction. These cells are available to assess the role of poly(ADP-ribosyl)ation DNA repair and in recovery from potential genotoxic agents, such as ADN and JP-8 without using non-specific chemical inhibitors. We have been successful with this approach and, during the last several years have published a number of studies describing the initial properties of these cell lines.

Ding, R. and Smulson, M: Depletion of Nuclear Poly(ADP-Ribose) Polymerase by Antisense RNA Expression: Influences on Genomic

The success of this technique with respect to lowering the poly ADPR response subsequent to strand breaks forms a basis to explore the role of this signal in DNA repair and cell survival in differing types of cells and tissues which may be at risk for exposure to **environmental hazards.**

In this work we thus utilized the ability of antisense expression in HeLa cells to deplete cellular levels of the enzyme to investigate several pleiotropic effects of PARP in strand break rejoining reactions. It was shown that: 1) Gene amplification was increased 2-3 fold in cells depleted of PARP compared to controls. 2) Chromatin structure was significantly altered in PARP-depleted cells, as indicated by reduced initiation and elongation of poly(ADP-ribose) chains attached to various nuclear protein acceptors altered poly ADPR of histone H1, and an increased susceptibility to micrococcal nuclease digestion. *These latter biochemical properties of PARP have been continually studied throughout the entire course of this project.* 3) The survival of PARP-depleted antisense cells exposed to the DNA alkylating agent methyl methane sulfonate and to nitrogen mustard was significantly reduced, relative to that of control cells (Fig. 18).

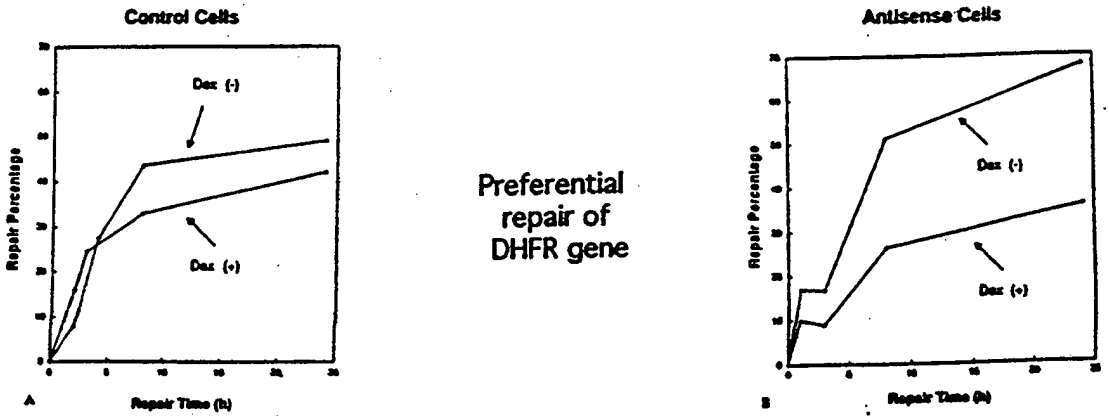


Figure 18

Ding, R., Stevnsner, T., Bohr, V., and Smulson, M. Effect of Depletion of Poly(ADP-ribose) Polymerase on Gene specific DNA Repair of Pyrimidine Dimers and Alkylation damage. Nucleic Acid Research, **22**:4620-4624, (1995).

Repair of alkylation adducts in DHFR gene caused by Nitrogen mustard (HN2) treatment - In **AIM II** of the renewal application, submitted to AFOSR several years ago, we proposed to establish this assay, to be used to test potentially hazardous agents such as ADN and JP-8. Thus in order to follow-through on the above observations and better describe the role of PARP in gene-specific repair, especially in preferential repair of active gene damage introduced by HN2, we analyzed the repair of the gene for DHFR in induced antisense cells were determined for DHFR gene in collaboration with Vil Bohr

ADN and JP-8. Thus in order to follow-through on the above observations and better describe the role of PARP in gene-specific repair, especially in preferential repair of active gene damage introduced by HN2, we analyzed the repair of the gene for DHFR in induced antisense cells were determined for DHFR gene in collaboration with Vil Bohr (NCI) using Southern hybridization analysis which was developed by Bohr while in the Hanawalt lab.

The data shows that induced antisense cells remove about 20 – 30% of alkylation adducts within 8 and 24h, respectively. However, the HN2-induced lesions were removed more efficiently within a 24 h period in the antisense cells with no Dex treatment. Thus, about 50% and 70% of repair occurred within 8 h and 24 h, respectively. The apparent distinction between repair patterns of induced and non-induced antisense cells indicates that an 80% reduction in PARP by antisense induction significantly inhibits the repair of HN2-induced DNA lesions in the active DHFR gene.

Smulson, M.E., Kang, V.H., Ntambi, J.M., Rosenthal, D.S., Ding, R., and Simbulan, C.M.G. Requirement for the Expression of Poly (ADP-ribose) Polymerase During the Early Stages of Differentiation of 3T3-L1 Preadipocytes, as Studied by Antisense RNA Induction. J. Biol. Chem. **270**(1):119-127 (1995).

A characterized stably transfected cell line of 3T3-L1 preadipocytes was established where PARP levels were manipulated by expression of antisense mRNA. Key observations made with this system were that PARP levels significantly increase, by both Western and transcript analysis, during the first day of the differentiation and then progressively decrease during the course of differentiation. In contrast, PARP levels do not increase during differentiation in antisense induced cells. Nor do the cells undergo the normal 2-3 rounds of cell division required during the initial 1-2 days of differentiation induction. The antisense cells occasionally progressed through one round of cell division; however, this resulted in extensive cytotoxicity and the cells proceeded to die, perhaps as a result of Okazaki fragments which are not able to be ligated and hence accumulate, or possibly through apoptosis.

Simbulan, C.M.G., Rosenthal, D.S., Hilz, H., Hickey, R., Malkas, L., Applegren, N., Wu, Y., Bers, G., Kang, V., and **Smulson, M.E.** The Expression of Poly(ADP-ribose) Polymerase during Differentiation-Linked DNA Replication Reveals that it is a Component of the Multiprotein DNA Replication Complex. Biochemistry, **35:**11622-11633, (1996).

As noted above we showed that 3T3-L1 preadipocytes exhibit a transient increase in PARP protein and activity, as well as an association of PARP with DNA polymerase α, within 12 to 24 h of exposure to inducers of differentiation; whereas, 3T3-L1 cells expressing PARP antisense RNA showed no increase in PARP and as noted above were unable to complete the round of DNA replication required for differentiation into

adipocytes. Flow cytometric analysis revealed that control 3T3-L1 cells progress through one round of DNA replication prior to the onset of terminal differentiation, whereas cells expressing PARP antisense RNA are blocked at the G_0/G_1 phase of the cell cycle. Confocal microscope image analysis of control S phase cells demonstrated that PARP was localized within distinct intranuclear granular foci associated with DNA replication centers.

On the basis of these results, purified replicative complexes from other cell types that had been characterized for their ability to catalyze viral DNA replication *in vitro* were analyzed for the presence of PARP. PARP exclusively co-purifies through a series of centrifugation and chromatography steps with core proteins of an 18-21S multiprotein replication complex (MRC) from human Hela cells, as well as with the corresponding mouse MRC from FM3A cells. The multienzyme complex were shown to contain DNA polymerases a and d, DNA primase, DNA helicase, DNA ligase, and topoisomerases I and II, as well as accessory proteins such as PCNA, RF-C, and RP-A. Finally, immunoblot analysis of MRCs from both cell types with monoclonal antibodies to poly(ADP-ribose) revealed the presence of approximately 15 poly(ADP-ribosyl)ated proteins, some of which were further confirmed to be DNA polymerase a, DNA topoisomerase I, and PCNA by immunoprecipitation experiments. These recent results suggested to us that PARP may play a regulatory role within the replicative apparatus as a molecular nick sensor controlling the progression of the replication fork, or modulates component replicative enzymes or factors in the complex by directly associating with them or by catalyzing their poly(ADP-ribosyl)ation.

Progress Report on use of JP8 Fuel throughout June, 1998

1. Initial collaborative Experiments with tissues Exposed to JP8 fuel by Drs. Witten and Harris.

Our work with JP 8 fuel began after we obtained tissues from animals treated with JP8 Dr. Mark Witten. Our initial study was to test the hypothesis that fuels induced various apoptotic markers. We initially (March 1997) received a variety of samples of spleen and Thymus cells from Dr. Harris's laboratory.

The first batch of the cells (exposed to JP 8 fuel at 1000 mg/m^3/hr) were as follows:

	Spleen cell #"s	Thymus cell #'s
Animal A Control	5×10^6	30×10^6
Animal B Control	6.6×10^6	30×10^6
Animal C Exposed	3×10^6	30×10^6
Animal D Exposed	2.8×10^6	40×10^6
Animal E Exposed	2.5×10^6	27×10^6
Animal F Exposed	6.6×10^6	50×10^6

We initially performed Apopain Assays on the above thymus cell extracts. We observed that spleen cell extracts were difficult to work with since they had become particulate upon addition of the lysis buffer. Accordingly the preliminary data for the apopain assay was not indicative of any significant result of fuel exposure as even the control cells appeared to show some apopain activity perhaps due to tissue preparation. Additionally the cells were treated with JP8 blend for 1 hour per day for 7 days, however, after treatment, they were left on ice for for 3 days before shipping (to achieve cell death). To determine apoptosis we realized it would be desirable in the future to analyse samples with exposures to JP 8 for periods of time longer than 1 hr. Unfortunately this was realised too late and the time of exposure was the same as above for all the subsequent shipments. This has subsequently been better clarified.

We also performed Hoechst staining to detect any nuclear fragmentation on these samples. The samples sent were fixed in formaldehyde, hence could be stained with the Hoechst stain. The nuclei in these cells looked intact.

2. Direct exposure of Jurkat cells in culture with water soluble compounds of JP8.

Since JP 8 is water insoluble, we collected the water soluble fraction by mixing equal volumes of JP8 blend and RPMI media, vortexing at high speeds and the samples were separated by centrifugation for 10 minutes at high speed.

In order to obtain samples with different doses of JP8 blend and for varying periods of time, we treated Jurkat cells with 1%, 5 % and 100% JP8 for 24 hours and

initially examined apoptosis by staining the cells with the Hoechst stain. The nuclei of cells treated with 1% and 5 % JP8 appeared smaller than normal. The cells treated with 100 % JP8 appeared apoptotic with nuclear fragmentation. This culture exposure appears to be promising and in the future other apoptotic markers will be examined. We will also utilize cultured lung cell lines, as well as primary lung cell cultures.

Progress Report Publications

Simbulan, C. M. G., Rosenthal, D. S., Hilz, H., Hickey, R., Malkas, L., Applegren, N., Wu, Y., Bers, G., Kang, V., and **Smulson, M. E.** The Expression of Poly(ADP-ribose) Polymerase during Differentiation-Linked DNA Replication Reveals that It Is a Component of the Multiprotein DNA Replication Complex. Biochemistry. **35:** 11622-11633 (1996).

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